Immunity
Commentary

CD28 Superagonists: What Makes the Difference in Humans?

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In 2006, a clinical trial with the CD28 superagonistic antibody TGN1412 in London turned into a catastrophe. Studies are beginning to unravel the difference between the human and preclinical animal response to the antibody.

Soon after the discovery of CD28 "superagonists" (CD28SAs), it was recognized that these antibodies can induce a preferential activation and expansion of immunosuppressive regulatory T cells in rat and mice (Lin and Hünig, 2003; Hünig, 2007) and that these particular properties can be used to ameliorate the onset, the progression, and the clinical course of experimental autoimmune diseases (for a review, see Hünig [2007]).

On the basis of these promising findings, it seemed possible to deploy CD28SAs for in vivo modulation of human autoimmune diseases. Consequently, after an enormous amount of preclinical data had been collected (including experiments involving cynomolgus and rhesus monkeys), a phase I clinical trial was conducted on March 13, 2006 at the Northwick Hospital in London, UK. During that trial, the humanized CD28 superagonist TGN1412 was administered to six healthy volunteers. Unfortunately, the trial turned into a catastrophe because TGN1412, unexpectedly, induced a rapid and massive cytokine storm that caused severe and life-threatening adverse effects in all six volunteers (Suntharalingam et al., 2006).

A tremendous amount of effort has been invested in understanding what went wrong at the trial, so what more do we know two years after the London incident that we didn't appreciate before?

Regulatory Measures Taken in the Aftermath of the TGN1412 Incident

Immediately after the TGN1412 incident, an investigation was initiated by the UK Medicines and Healthcare products Regulatory Agency (MHRA) that had approved the TGN1412 trial. This investigation came to the conclusion that the adverse incidents did not involve errors in the manufacture of TGN1412 or in its formulation, dilution, or administration to trial participants and that therefore unpredicted biological action of the drug in humans accounted for the devastating effects in the trial participants (http://www.mhra.gov.uk/home/idcplg?IdcService=GET_FILE&dDocName=CON2023821&RevisionSelectionMethod=LatestReleased). This notion raised concerns regarding whether currently available knowledge is sufficient to tame superantibody drugs.

A number of European agencies, including the French, British, and German, formulated recommendations to improve safety of first-in-human clinical trials (for review, see Schneider and Kalinke [2007]). The Expert Scientific Group convened in the UK to further investigate the TGN1412 incident and to develop recommendations to minimize risks associated with phase I clinical trials of innovative medicinal products. Repetitions of preclinical experiments, including nonhuman primate studies, commissioned by the Expert Scientific Group recapitulated the findings as summarized in the original investigator's brochure of TeGenero (the company that developed TGN1412). These results and a total of 22 recommendations addressing the transition from preclinical to clinical development, the clinical development, regulatory aspects, and future perspectives were published at the end of 2006 in a comprehensive final report (Duff, 2006). On the basis of the above considerations, the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMEA) developed a guideline on strategies to identify and mitigate risks for first-in-human clinical trials that is in effect since September 2007 and that is not only applicable to mAbs and other biologicals but also to conventional medicinal products (EMEA, 2007).

Nevertheless, although effective regulatory measures were undertaken in a timely manner that definitely further improve overall safety of phase I clinical trials, the most critical scientific question that was put forward in one of the first reports after the TGN1412 incident, the investigation report by MHCR, is still only insufficiently solved, i.e., why the resulting TGN1412 activity seen in humans was not predicted from apparently adequate preclinical testing.

In order to solve this puzzle, it will be mandatory to carefully (re)assess functional responses of CD28SA-stimulated human, rat, and monkey T cells by biochemical, cell-biology, and molecular-biology means. Indeed, if the species-specific differences are understood on the molecular level, it might be possible to avoid incidents such as the one in London in the future and to predict potential adverse effects already in the preclinical phase of drug development.

Recent studies have now begun to shed some light into the question of why rodent and monkey T cells behave differently when compared to human T lymphocytes upon CD28SA stimulation.

T Cell Activation by CD28 Superagonists

One paradigm of T cell activation is the requirement of two simultaneously applied signals (Schwartz, 1992): The first signal is antigen dependent and is mediated

via the T cell receptor (TCR), whereas the second signal is antigen independent and is provided by costimulatory receptors, such as CD28. In vivo, the first signal is mediated by peptide-MHC complexes that bind to the TCR, whereas the second signal is initiated after CD28 has been engaged by its natural ligands, CD80 and CD86, both of which are expressed on mature dendritic cells.

Under in vitro conditions, the second signal can be mimicked by so-called conventional CD28 antibodies (Acuto and Michel, 2003). According to the above paradigm, these reagents support T cell activation only in combination with a primary, TCR-mediated, stimulus (e.g., provided by TCR antibodies). Approximately ten years ago, a new group of CD28 antibodies, now called CD28 superagonists (CD28SA) or mitogenic CD28 mAbs, has been described (Bischof et al., 2000; Dennehy et al., 2007; Dennehy et al., 2003; Tacke et al., 1997). In contrast to conventional CD28 antibodies, CD28 superagonists are capable of triggering the activation of rat, mouse, and human T lymphocytes upon binding to CD28 alone. How CD28SAs induce polyclonal T cell activation (and thus provide signal one and two simultaneously) is still not completely understood.

Signaling Properties of CD28SAs

Before the London trial, the signaling properties of superagonistic CD28 mAbs had been most extensively studied in rat and mouse T cells (Dennehy et al., 2003; Lühder et al., 2003). Only recently, signaling signatures of anti-human CD28 superagonists have also been assessed (Dennehy et al., 2007; Sester et al., 2007; Stebbings et al., 2007; Waibler et al., 2008).

Overall, it appears as if the signaling events generated by anti-rat and anti-mouse versus anti-human CD28SAs are rather similar. For instance, signaling via both anti-rat and anti-human CD28SAs depends on Src-family protein tyrosine kinases and activation of the SLP76 signalosome (which is required for inducing transmembranous calcium flux and consists of the adaptor protein SLP76, the nucleotide exchange factor Vav, the Tec-kinase Itk, and phospholipase Cγ). Further, anti-rat and anti-human CD28SAs activate protein kinase Cθ and ras and Erk kinases, as well as the transcription factors NF-ATc1 and NF-κB (Bischof et al., 2000; Dennehy et al., 2003; Sester et al., 2007; Waibler et al., 2008). How all these signals are initiated at the plasma membrane is still not completely understood. One hypothesis is that CD28SAs might induce massive clustering of CD28, thereby generating signaling patches within the membrane. However, at least by conventional confocal microscopy, the clusters that are produced by CD28SAs cannot be distinguished from those generated by conventional CD28 antibodies (Sester et al., 2007 and our own unpublished data). Hence, more sophisticated microscopic and/or biochemical studies are required to assess this question.

Recently, it was shown that in humans and rats, the signaling capacity of CD28SAs depends on the expression of a functional TCR (Dennehy et al., 2007; Waibler et al., 2008). On the basis of these rather surprising findings, it was hypothesized that CD28 superagonists induce T cell activation by amplifying tonic signals that emanate from the unligated TCR and, further, that this amplification occurs at the level of the SLP76 signalosome (Dennehy et al., 2007). However, CD28SA-mediated signaling could also feed into the TCR pathway upstream of this complex, e.g., at the level of the transmembrane adaptor molecule LAT or the protein tyrosine kinase ZAP70 (Waibler et al., 2008).

In rats, a particular binding site for the adaptor protein Grb2 in the cytoplasmic domain of CD28 appears mandatory for CD28SA signaling because mutation of this site impairs calcium flux and interleukin-2 secretion in response to CD28SAs (Dennehy et al., 2007). Biochemical experiments further suggested that these defects result from an insufficient activation of Itk and Vav within the SLP76 signalosome. How CD28 couples to Itk and Vav and whether anti-human CD28SAs employ a similar mechanism to activate T cells is as yet unclear.

One important point that needs clarification relates to the role of PI3 kinase (PI3K) in anti-human versus anti-rat CD28SA-induced cytokine production. In rat T cells, mutation of the PI3K-binding site in the cytoplasmic domain of CD28 does not affect CD28SA-mediated induction of calcium flux and IL-2 synthesis (Dennehy et al., 2007). In contrast, pharmacologic inhibition of PI3K completely abrogates CD28SA-mediated interferon-γ (IFN-γ) and IL-2 production of human T cells in vitro (Waibler et al., 2008). Collectively, these data might suggest a different requirement for PI3K in CD28SA-mediated cytokine production and proliferation in human versus rat T cells. It will be of great importance to elucidate this question because, if the differences between the human and rodent system could be confirmed, this might explain some of the particular functional effects of anti-human CD28SAs.

We recently showed that both TGN1412 as well as a commercially available CD28SA, ANC28.1, induce an extremely sustained transmembranous calcium flux in human T cells (Waibler et al., 2008). Moreover, the amounts of IFN-γ and IL-2 that are secreted by ANC28.1-stimulated human T cells in vitro strictly correlate with the duration of calcium flux. Hence, it seems as if the cytokine storm that was generated by TGN1412 is strongly associated with an extremely sustained transmembranous calcium flux. How this flux is generated by TGN1412 and ANC28.1 is currently unclear. Unfortunately, it is also unknown whether anti-rat and anti-mouse CD28SAs induce a similarly shaped calcium response as anti-human CD28SAs (Table 1). Indeed, so far only short-term kinetics had been performed in all experiments dealing with CD28SA-mediated calcium flux in rat and mouse T cells (see e.g., Dennehy et al., 2003).

Table 1. Potential Species-Specific Differences in CD28SA-Mediated Signaling

Species	Dependency on TCR Expression	Calcium Flux >2 hr	PI3K Requirement	Expression of Siglecs
Rat	yes	not assessed	unclear	not systematically assessed
Cynomolgus	not yet assessed	yes, but very weak	not assessed	not systematically assessed
Human	yes	yes, >6 hr	yes	lost during evolution

Human versus Monkey T Cells

A major question regarding the London trial is why two nonhuman primate

species, i.e., cynomolgus and rhesus monkeys, did not show obvious adverse effects upon TGN1412 treatment. This is particularly surprising in light of the fact that the extracellular domains of cynomolgus and rhesus monkey and human CD28 are completely conserved (Hanke, 2006; Waibler et al., 2008). Further, the overall numbers of CD28 molecules that are expressed by cynomolgus and rhesus monkey T cells appear to be similar, if not identical, to human T cells. In addition, the affinity of TGN1412 toward human and monkey CD28 appears to be comparable (Hanke, 2006; Waibler et al., 2008).

Principally two possibilities could be envisaged (which are not mutually exclusive) to explain differential in vivo responses in humans and nonhuman primates. The first would be that the magnitude of the TGN1412 response elicited in monkey T cells is much lower than that in human T cells (quantitative model). Alternatively (or additionally), TGN1412 stimulation of monkey T cells might not activate the same intracellular signaling pathways as it does in human cells (qualitative model) and, consequently, might not induce cytokine synthesis.

Unfortunately the "qualitative model" is somewhat difficult to prove because the numbers of T cells that can be isolated from rhesus and cynomolgus monkeys are too low to perform well-conducted biochemical studies. So that this problem is avoided, it would be necessary to establish new methods such as flow-cytometry assays that allow detection of phosphorylation events and the correlation with the activation of various intracellular signaling pathways in monkey T cells.

With regard to the "quantitative model," we showed that the TGN1412-mediated calcium response of monkey T cells, albeit being also sustained, is much lower compared to human T lymphocytes (Waibler et al., 2008). Moreover, an independently performed study came to the conclusion that monkey peripheral blood mononuclear cells (PBMCs), unlike human cells, do not produce detectable amounts of cytokines in vitro when activated by CD28SAs (Stebbings et al., 2007). Hence, it appears as if monkey T cells are less responsive toward CD28SA stimulation than human T cells.

In this context, it was discussed whether the TGN1412-mediated response in monkeys might be lower because the animals are kept under more "sterile" conditions compared to humans and that therefore the monkey T cell population might be more "naive." However, there is currently no convincing evidence supporting this notion. Indeed, monkeys are not kept under specified pathogen-free (SPF) conditions and they also receive "normal" food. Moreover, the CD28SA-mediated calcium flux and the in vitro-inducible cytokine production do not appear to differ dramatically between naive and memory human T cells (Sester et al., 2007; Waibler et al., 2008). In summary, particular housing conditions or subtle difference in the composition of T cell subsets are unlikely to explain the reduced responsiveness of monkey T cells toward TGN1412.

What else could explain the quantitatively different CD28SA-response of human versus monkey T cells? As reported above, the extracellular domains of monkey and human CD28 are identical on the amino acid level, and also the TGN1412 binding of CD28 expressed by human and nonhuman primate T cells is comparable. However, sequence comparison revealed three amino acid exchanges in the corresponding transmembrane domains between humans and nonhuman primates (Waibler et al., 2008). These might influence the lateral interactions of CD28 with other signal-transducing molecules within the cell membrane and thus alter the outcome of CD28SA stimulation. For assessing this point, it will be necessary to reconstitute CD28-deficient T cells with CD28-swapping mutants carrying the human or monkey transmembrane domains to subsequently compare CD28SA-mediated responses in the two types of cells.

An alternate, yet attractive, hypothesis that could explain the quantitatively different reactivity of monkey versus human T cells toward TGN1412 stimulation relates to the differential expression of a family of molecules called Siglecs (Table 1). Siglecs are integral membrane receptors belonging to the immunoglobulin superfamily. Within the cytoplasmic tails, some Siglecs carry ITIMs (immunoreceptor-tyrosine-based inhibition motif). Upon tyrosine phosphorylation (which occurs by protein tyrosine kinases of the Src-family), the ITIMs recruit tyrosine phosphatases such as SHP1 or SHP2 to the plasma membrane, which in turn dephosphorylate key tyrosine residues in other signaling proteins (Crocker et al., 2007).

Strikingly, Siglecs are barely detectable on human T cells, whereas T cells from chimpanzees express various members of these receptors (Nguyen et al., 2006). Moreover, ectopic expression of Siglec-5 (the major Siglec expressed by chimpanzee T cells) on human T cells impairs CD3-mediated responses, whereas the opposite is true if Siglec-5 is cleared from the surface of chimpanzee T cells (Nguyen et al., 2006). These data would support the idea that an evolutionary loss of Siglec expression on human T cells lowers the signaling thresholds that are required to activate the intracellular signaling machinery. However, it remains to be determined whether rhesus and/or cynomolgus monkey T cells express Siglecs and whether alterations in Siglec expression not only affect TCR-mediated T cell responses in chimpanzees and other monkeys but also those induced by CD28 superagonists.

The Fc-Receptor Issue

Many antibody functions are mediated via Fc-receptor binding. In the case of TGN1412, the constant part was expressed as an IgG4. The observation that calcium flux and cytokine secretion require crosslinking of the TGN1412 (Waibler et al., 2008) raises the question of which Fcγ receptor expressing cell types could have provided TGN1412 crosslinking in vivo and whether such crosslinking is similarly observed in nonhuman primates. In this regard, it was recently demonstrated that in vitro cytokine production of PBMC can be induced by TGN1412 in the presence of human endothelial cells (Stebbings et al., 2007). However, sequence analysis suggests that human and nonhuman primate Fcγ receptors share a high degree of similarity (Presta and Namenuk, 2005). Hence, it is unclear whether endothelial cells of human and nonhuman primate origin can similarly crosslink CD28SA and, if so, whether such crosslinking plays a critical role for cytokine production in vivo and, further, which Fc-receptor is involved.

CD28SA and Regulatory T Cells

During the preclinical phase CD28SAs had been extensively tested in rodents (for a review, see Hünig [2007]) in which administration of CD28SAs induced

a transient lymphocytosis followed by a preferential expansion of natural regulatory T (Treg) cells. Proinflammatory cytokines such as IFN-γ and IL-2 were not detected in the animals. Rather, a production of the anti-inflammatory cytokines IL-10 and IL-4 was found (Hünig, 2007). The lack of production of proinflammatory cytokines was believed to be due to the fact that in rodents, the expansion and activation of Treg cells occur so quickly that they suppress cytokine production by conventional $CD4^+$ T cells (which clearly also become activated by CD28SAs) before it reaches amounts that could cause physiological changes. A recent report extended this hypothesis by showing that in rats, the CD28 superagonist JJ316 induces two waves of T cell activation in vivo: A fast and transient wave (<48 hr) that affects both conventional and Treg cells and a second, delayed wave (>48 hr) that exclusively results in the activation of Treg cells (Müller et al., 2008). Surprisingly, although during the first phase conventional T cells strongly upregulate mRNA for proinflammatory cytokines, serum levels remain low (Müller et al., 2008).

As we have learnt from the London trial, this mode of CD28SA stimulation does not hold true for the in vivo situation in humans. Moreover, despite differences in the experimental design, three recent studies showed production of substantial amounts of proinflammatory cytokines after in vitro CD28SA stimulation of human T cells or PBMCs (Sester et al., 2007; Stebbings et al., 2007; Waibler et al., 2008). Although the experimental settings in two of these studies (Sester et al., 2007; Waibler et al., 2008) might not appropriately reflect the situation in vivo (because cytokine responses were only assessed after crosslinking of the CD28SAs by means of secondary antibodies), it is likely that even if anti-human CD28SAs induce the activation or expansion of natural human Treg cells (which still has to be demonstrated), this appears to occur either with a much slower kinetics or with much lower efficiency compared to rodents. In both cases, the result is the same, namely a predominant production of proinflammatory cytokines by CD28SA-stimulated human $CD4^+$ T cells.

The implication from these in vivo and in vitro data goes far beyond CD28SA signaling. Indeed, the London incident provided additional evidence that signals leading to activation and/or expansion of Treg cells presumably show subtle differences that eventually may result in dramatically different outcomes in the rodent and human system. Hence, data that were obtained in the mouse system cannot be transferred to the human system without rigorous experimental verification. One exciting approach to address this problem might be new developments in the field of humanized mice. Interestingly, experiments in that direction had also been performed with a mouse anti-human CD28 superagonistic antibody (Legrand et al., 2006). However, from today's perspective, the relevant questions had not been addressed in that study, i.e., cytokine responses were not analyzed in the anti-CD28SA-treated humanized mice.

Conclusions

The currently available experimental data suggest that only minor differences in the organization and regulation of T cell responses account for dramatic species-specific differences that are observed after CD28 superagonistic T cell activation and that only in-depth experimental analysis might allow solving this puzzle. Besides providing insights into the species-specific organization of central signaling pathways, such experiments could also help to better validate concepts and working models during the preclinical phase of drug development. In addition, to make drug development safer, interdisciplinary activities need to be strengthened in the future. For example, systems-biology approaches might help to predict potential adverse effects of novel drugs in silico which then could be screened for by designing appropriate experiments. Moreover, valuable information about novel drugs is usually deposited in data collections of single pharmaceutical companies, but in general this information is not easily accessible in public domains.

Nevertheless, it should be kept in mind that even the most sophisticated and extensive in vitro, in vivo, and in silico analyses may again fail to predict reactivity of agonistic biologicals in humans. Thus, the most important safety measure learned from the TGN1412 trial appears to be the application of the minimal anticipated biological effect level (MABEL) instead of the nonobserved adverse effect level (NOAEL) for first-in-human dose calculations. The advantage of the MABEL approach is that it is based on straightforward parameters such as receptor occupancy and leads to a much lower entry dose compared to the NOAEL calculation. Especially in the case of reagents with agonistic properties, this might substantially increase the safety of first-in-human studies.

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